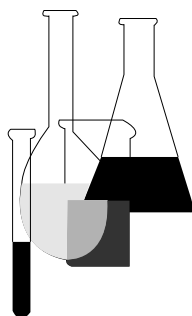




Ecological Effects Test Guidelines

OPPTS 850.6800 Modified Activated Sludge, Respiration Inhibition Test for Sparingly Soluble Chemicals



“Public Draft”

INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Public Draft Access Information: This draft guideline is part of a series of related harmonized guidelines that need to be considered as a unit. *For copies:* These guidelines are available electronically from the EPA Public Access Gopher (gopher.epa.gov) under the heading “Environmental Test Methods and Guidelines” or in paper by contacting the OPP Public Docket at (703) 305-5805 or by e-mail: guidelines@epamail.epa.gov.

To Submit Comments: Interested persons are invited to submit comments. By mail: Public Docket and Freedom of Information Section, Office of Pesticide Programs, Field Operations Division (7506C), Environmental Protection Agency, 401 M St. SW., Washington, DC 20460. In person: bring to: Rm. 1132, Crystal Mall #2, 1921 Jefferson Davis Highway, Arlington, VA. Comments may also be submitted electronically by sending electronic mail (e-mail) to: guidelines@epamail.epa.gov.

Final Guideline Release: This guideline is available from the U.S. Government Printing Office, Washington, DC 20402 on *The Federal Bulletin Board*. By modem dial 202-512-1387, telnet and ftp: fedbbs.access.gpo.gov (IP 162.140.64.19), or call 202-512-0135 for disks or paper copies. This guideline is also available electronically in ASCII and PDF (portable document format) from the EPA Public Access Gopher (gopher.epa.gov) under the heading “Environmental Test Methods and Guidelines.”

OPPTS 850.6800 Modified activated sludge, respiration inhibition test for sparingly soluble chemicals

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are 40 CFR 795.170 (proposed in the FEDERAL REGISTER of May 15, 1986 (51 FR 17880)) and OECD guideline 209, Activated Sludge, Respiration Inhibition Test.

(b) **Introductory information**—(1) **Prerequisites.** Water solubility, vapor pressure, information on the structural formula of the test substance will be useful.

(2) **Recommendation.** Activated sludge may contain potentially pathogenic organisms and should be handled with care.

(3) **Standard documents.** This test guideline has been based on the references in paragraphs (f)(1) through (f)(6) of this guideline.

(c) **Method**—(1) **Introduction, purpose, scope, relevance, application, and limits of test.** (i) The method described in this test guideline assesses the effect of a test substance on microorganisms by measuring the respiration rate under defined conditions in the presence of different concentrations of the test substance. The method is based on that described by the Ecological and Toxicological Association of the Dyestuffs Manufacturing Industry, in which activated sludge obtained from a sewage treatment plant is used as the microbial source.

(ii) The purpose of this test guideline is to provide a rapid screening method whereby substances which may adversely affect aerobic microbial treatment plants can be identified and to indicate suitable noninhibitory concentrations of test substances to be used in biodegradability tests.

(iii) A range-finding test may precede a definitive test. It provides information about the range of concentrations to be used in the main test.

(iv) Two controls without test substance are included in the test design, one at the start and the other at the end of the test series. Each batch of activated sludge should also be checked using a reference substance.

(2) **Definitions.** The definitions in section 3 of the Toxic Substances Control Act (TSCA) and the definitions in 40 CFR Part 792—Good Laboratory Practice Standards apply to this test guideline. The following definitions also apply to this test guideline.

Respiration rate. The oxygen consumption of aerobic sludge or wastewater microorganisms, generally expressed as milligrams of O₂ per liter per hour.

Effective concentration (EC50). In this test guideline, the concentration of the test substance at which the respiration rate is 50 percent of that shown by the control under conditions in this guideline.

(3) **Reference substances.** 3,5-Dichlorophenol (a known inhibitor of respiration) should be used as a reference substance and tested for EC50 on each batch of activated sludge as a means of checking that the sensitivity of the sludge is not abnormal.

(4) **Principle of the test method.** The respiration rate of an activated sludge fed with a standard amount of synthetic sewage feed is measured after a contact time of 30 min or 3 h, or both. The respiration rate of the same activated sludge in the presence of various concentrations of the test substance under otherwise identical conditions is also measured. The inhibitory effect of the test substance at a particular concentration is expressed as a percentage of the mean respiration rates of two controls. An EC50 value is calculated from determinations at different concentrations.

(5) **Conditions for the validity of the test.** (i) The test results are valid if the two control respiration rates are within 15 percent of each other.

(ii) The EC50 (3–h) of 3,5-dichlorophenol must be in the accepted range of 5 to 30 mg/L.

(d) **Description of the test procedure—(1) Preparations—(i) Equipment.** Normal laboratory equipment and especially the following is necessary:

(A) Measuring apparatus (flat-bottom flask, stirrer bar, magnetic stirrer, oxygen electrode, and recorder).

(B) Aeration device.

(C) pH electrode and measuring equipment.

(D) Oxygen electrode.

(2) **Solutions of the test substance.** (i) Solutions of the test substance are freshly prepared at the start of the study using a stock solution. A stock solution concentration of 0.5 g/L is appropriate if the procedure recommended below is followed. (Note: A solution of 3,5-dichlorophenol can be conveniently prepared by dissolving 0.5 g of 3,5-dichlorophenol in 10 mL of 1N NaOH, diluting to approximately 30 mL with distilled water, adding, while stirring, 1N H₂SO₄ to the point of incipient precipitation,

and finally diluting the mixture to 1 L with distilled water. The pH should then be in the range 7 to 8.)

(ii) If the test substance is not sufficiently soluble to allow preparation of a concentrated stock solution in water, it should be added directly to test vessels or, alternatively, as a concentrated stock solution in an organic solvent. Direct addition is recommended. If an organic solvent is used, the solvent must neither significantly inhibit nor contribute to respiration. Further, a control containing activated sludge and solvent but no test substance is required.

(3) **Test concentrations.** At least five concentrations, spaced by a constant factor preferably not exceeding 3.2, should be used.

(4) **Synthetic sewage feed.** (i) A synthetic sewage feed is made by dissolving the following amounts of substances in 1 L of water:

- (A) 16 g peptone.
- (B) 11 g meat extract.
- (C) 3 g urea.
- (D) 0.7 g NaCl.
- (E) 0.4 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$.
- (F) 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$.
- (G) 2.8 g K_2HPO_4 .

(ii) This synthetic sewage is a 100-fold concentrate of that described in the OECD Technical Report (June 11, 1976) ‘‘Proposed method for the determination of the biodegradability of surfactants used in synthetic detergents,’’ with dipotassium hydrogen phosphate added.

(5) **Test system.** Activated sludge from a sewage treatment plant is normally used as the microbial inoculum for the test. Where possible, activated sludge should be obtained from a sewage works treating predominantly domestic sewage. If this is not possible, the activated sludge may be obtained from sewage works treating predominantly industrial waste water but used only following deadaptation. Even so, results obtained with activated sludge from works treating industrial waste waters may be atypical. On return to the laboratory the sludge is washed, if necessary, with tap water or an isotonic solution. After centrifuging the supernatant is decanted. This procedure is repeated three times. A small amount of the washed sludge is weighed and dried. From this result the amount of wet sludge can be calculated which must be suspended in water in order to obtain an activated sludge with a mixed liquor suspended solids level of 4 g/L (± 10 percent). This level gives a concentration of 1.6 g/L in the test medium if the procedure recommended below is followed. If the

sludge cannot be used on the day of collection, 50 mL synthetic sewage is added to each liter of the activated sludge prepared as described above; this is then aerated overnight at 20 ± 2 °C. It is then kept aerated for use during the day. Before use the pH is checked and buffered, if necessary to pH 6.0 to 8.0 using sodium bicarbonate solution. The mixed liquor suspended solids should be determined as described in the preceding paragraph. If the same batch of sludge is required to be used on subsequent days (maximum 4 days), a further 50 mL of synthetic sewage feed is added at the end of each working day.

(6) **Test conditions.** The following conditions apply to the test system:

(i) Duration/contact time: 30 min and/or 3 h, during which aeration takes place.

(ii) Vessels: Beakers are suitable.

(iii) Water: Drinking water (dechlorinated if necessary).

(iv) Air supply: Clean oil-free air. Air flow 0.5 to 1 L/min.

(v) Measuring apparatus: Flat-bottom flask such as a BOD-flask.

(vi) Oxygen meter: Polarographic oxygen electrode, connectable to a potentiometric recorder (200 mv range).

(vii) Nutrient solution: Synthetic sewage feed. (See paragraph (d)(4)(i) of this guideline.)

(viii) Test substance: The test solution is freshly prepared at the start of the test.

(ix) Reference substance: For example 3,5-dichlorophenol (at least 3 concentrations).

(x) Controls: Inoculated sample without test substance.

(xi) Temperature: 20 ± 2 °C.

(7) **Performance of the test.** A suggested experimental procedure which may be followed for both the test and reference substance for the 3-h contact period is:

(i) Several vessels (e.g., 1-L beakers) are used.

(ii) At time “0” 16 mL of the synthetic sewage feed is made up to 300 mL with water. Microbial inoculum (200 mL) is added and the total mixture (500 mL) poured into a first vessel (first control C1). Aeration at 0.5 to 1 L/min is commenced using a Pasteur pipet as aeration device.

(iii) (A) At time “15 min” (15 min is an arbitrary, but convenient, interval) the process described in paragraph (d)(7)(ii) of this guideline is repeated, except that 100 mL of the test substance stock solution is added to the 16 mL of synthetic sewage before adding water to 300 mL and microbial inoculum to make a volume of 500 mL. This mixture is then poured into a second vessel and aerated as described in paragraph (d)(7)(ii) of this guideline. This process is repeated at 15-min intervals with different volumes of the test substance stock solution to give a series of vessels containing different concentrations of the test substance. Finally, a second control (C2) is prepared.

(B) If the test substance is not sufficiently soluble to allow addition from a stock solution in water, the appropriate proportion of the 100 mL volume of test substance stock solution referred to in paragraph (d)(2)(i) is replaced with water. For example, if 10 mL of an insoluble liquid test substance or solvent containing test substance is added directly to the test vessels, 90 mL of water is added. If insoluble solid test substance is added to the test vessel, 100 mL of water is added. If test substance is added as a stock solution in an organic solvent, a third control, containing the appropriate amount of solvent plus water to a total of 100 mL, is required, in addition to the series of test vessels containing different concentrations of the test substances.

(iv) After 3 h the contents of the first vessel are poured into the measuring apparatus and the respiration rate is measured over a period of up to 10 min; the measuring can also be carried out directly in the vessel.

(v) This determination is repeated on the contents of each vessel at 15-min intervals, in such a way that the contact time in each vessel is three h.

(A) The reference substance is tested on each batch of microbial inoculum in the same way. A different regime (e.g., more than one oxygen meter) will be necessary when measurements are to be made after 30 min of contact.

(B) If measurement of the chemical oxygen consumption is required, further vessels are prepared containing test substance, synthetic sewage feed and water, but no activated sludge.

(8) **Observations.** Oxygen consumption is measured and recorded after an aeration time of 30 min and/or 3 h contact time.

(e) **Data and reporting—(1) Treatment of results.** (i) The respiration rate is calculated from the recorder trace as milligrams of O_2 /L/h between approximately 6.5 mg O_2 /L and 2.5 mg O_2 /L, or over a 10-min period when the respiration rate is low. The portion of the respiration curve over which the respiration rate is measured should be linear. In order to calculate the inhibitory effect of a test substance at a particular concentra-

tion, the respiration rate is expressed as a percentage of the mean of the two control respiration rates:

Formula:

$$[1 - 2 R_s / (R_{C1} + R_{C2})] \times 100 = \text{percent inhibition}$$

where

R_s = oxygen-consumption rate at tested concentration of test substance

R_{C1} = oxygen-consumption rate, control 1

R_{C2} = oxygen-consumption rate, control 2

(ii) If the respiration rates of the two controls are not within 15 percent of each other or the EC50 (3h) of the reference substance is not in the accepted range (5 to 30 mg/L for 3,5-dichlorophenol), the test is invalid and must be repeated. The percent inhibition is calculated at each test concentration using the formula shown above. The percent inhibition is plotted against concentration on log-normal (or log-probability) paper and an EC50 value derived. Ninety five percent confidence limits for the EC50 values can be determined using standard procedures. In view of the variability often observed in the results, it is recommended that the results be expressed in orders of magnitude, e.g., less than 1, 1 to 10, 10 to 100, etc. (in mg/L).

(2) **Interpretation of results.** The EC50 value should be regarded merely as a guide to the likely toxicity of the test substance either to activated sludge sewage treatment or to wastewater microorganisms, since the complex interactions occurring in the environment cannot be accurately simulated in a laboratory test.

(3) **Test report.** The test report should include the following information.

(i) Test substance: Chemical identification data.

(ii) Test system: Source, concentration and any pretreatment of the activated sludge.

(iii) Test conditions: Test temperature, test duration, reference substance and its measured EC50, and abiotic oxygen uptake, if any.

(iv) Results: All measured data; inhibition curve and method for calculation of EC50; EC50, and if possible, 95 percent confidence limits; EC20 and EC80; all observations and any deviations from this test guideline which could have influenced the result.

(f) **References.** The following references should be consulted for additional background material on this test guideline.

(1) International Standard ISO/TC 147/SC 5/WC 1, N53 No. D (June 1981).

(2) Broecker, B. and Zahn, R., *Water Research* 11:165 (1977).

(3) Brown, D. et al. *Chemosphere* 10:245 (1981).

(4) ETAD (Ecological and Toxicological Association of Dyestuffs Manufacturing Industries) Recommended Method No. 103, also described by:

(5) Robra, B. *Wasser/Abwasser* 117:80 (1976).

(6) Schefer, W. *Textilveredlung* 6:247 (1977).